Journal of Advanced Medical and Dental Sciences Research

@Society of Scientific Research and Studies

Journal home page: www.jamdsr.com doi: 10.21276/jamdsr UGC approved journal no. 63854

(e) ISSN Online: 2321-9599; (p) ISSN Print: 2348-6805 SJIF (Impact factor) 2017= 6.261; Index Copernicus value 2016 = 76.77

Original Article

Detection of Methicillin resistance among nasal isolates of staphylococcus aureus in HIV patients: An evaluation of three different screening methods

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ABSTRACT

Background: HIV is a well established risk factor for nasal colonization of staphylococcus aureus. MRSA is substantial cause of morbidity and mortality among HIV infected patients worldwide. Hence, Rapid and accurate method for detection of Methicillin resistant *Staphylococcus aureus* (MRSA) is an important role of clinical microbiology laboratories to avoid treatment failure and to control the endemicity of MRSA. The aim of this study was to compare three conventional methods against the molecular method to evaluate the best phenotypic method for route laboratory. **Methods**: A total of 440 nasal swab samples from 220 HIV positive cases and 220 HIV negative controls were processed, among which 144 isolates of *S. aureus* were obtained. Methicillin resistance was determined by oxacillin disc diffusion, cefoxitin disc diffusion the Oxacillin screen agar test and mecA gene detection by PCR**.** **Result**: Out of 144 nasal isolates of S. aureus, 16, 20 and 21 isolates were identified as MRSA based on Oxacillin disc diffusion method, Oxacillin screen agar method, Cefoxitin disc diffusion method respectively. In all phenotypic methods, Cefoxitin disc diffusion test better correlates with gold standard mecA gene PCR method for detection of MRSA having 100% specificity (95% CI; 97.05% -100.00%), 100% sensitivity (95% CI; 83.89% -100.00%) and 100% accuracy (95% CI; 97.47%- 100.00%). **Conclusion: -** Our study revealed that cefoxitin disk diffusion method had a high sensitivity, specificity and accuracy comparative to other phenotypic methods for detection of MRSA. It is also easy to perform and cost effective as compared to other method.

Keywords: Nasal isolates, *S. aureus,* MRSA, Cefoxitin disc diffusion.

Received: 12 September 2018 Revised: 23 September 2018 Accepted: 25 September 2018

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This article may be cited as: Diwakar MK, Gupta P, Goyal A. Detection of Methicillin resistance among nasal isolates of staphylococcus aureus in HIV patients: An evaluation of three different screening methods. J Adv Med Dent Scie Res 2018;6(10):1-5.

INTRODUCTION:

Staphylococcus aureus is one of the most important bacterial pathogen in the hospital as well as in community settings.[1] MRSA infections are always a therapeutic challenge for physicians because of the limited choice of therapeutic options available and due to the possibility of multi-drug drug resistance of the MRSA.[2] The continuous increase in the MRSA isolates in this geographical region makes the scenario more drastic.[3] Carriage of S. aureus has been identified as a potent risk factor for the development of staphylococcal infections in various settings. This has been observed extensively in patient with immunodeficiency such as HIV infection.[4, 5] Staphylococcus aureus infections account for significant morbidity in human immunodeficiency virus (HIV)-infected patients.[6] The high prevalence of nasal carriage staphylococcus aureus in this geographical region is matter of concern. Expression of Methicillin resistance in the laboratory depends upon environmental conditions like temperature, pH, incubation time, salt concentration in the medium, sucrose to culture medium, incubation at 30^0C and passage in the presence of β-lactam antibiotics which enhances the expression of resistance.[7-9] Conditional expression of PBP2a antigen may cause ambiguity in susceptibility testing.[10] Methicillin resistance often expressed heterogeneously and cause a diagnostic difficulty as only 1 in 10^4 to 10^7 cells of the population found phenotypically resistant.[11]

These factors emphasize the need to choose a rapid, accurate and sensitive method to detect Methicillin resistance staphylococci which is not dependent on growth conditions. The conventional methods to detect

MRSA in the laboratory include Oxacillin disk diffusion test, Oxacillin screen agar method and cefoxitin disc diffusion test. Methicillin resistance is caused by the presence of *mec- A* gene, which encodes a low affinity penicillin binding protein PBP2a or PBP2' which has a low affinity for β-lactam antibiotics. Therefore, presence of *mec-A* gene confirms Methicillin resistance in *Staphylococci*.[12] Various diagnostic tools that improve the turnaround time for the detection of MRSA have been described. Polymerase chain reaction (PCR) is considered the **gold standard.** [13] It is rapid, reliable, highly sensitive and specific, but is expensive and needs expertization. it is not yet available in all diagnostic laboratories, therefore phenotypic methods still remain as methods of choice in the resource limited settings. [14]

Hence, Rapid, cost effective and accurate method for detection of Methicillin resistant *Staphylococcus aureus* (MRSA) is essential for effective antimicrobial therapy to avoid treatment failure. The aim of this study was to compare three conventional methods against the molecular method to evaluate the best phenotypic method for route laboratory.

MATERIALS AND METHODS:

Two hundred twenty patients; freshly diagnosed with HIV infection, attending the ICTC centre at a tertiary care hospital were enrolled as case group after obtaining informed consent. Similarly, 220 healthy people willing to participate in the study were included as control group in this study. Patients having diabetes, cancer, haemodialysis and other conditions leading to immunodeficiency were excluded from the study. Anterior nares swab sample from all the study population were collected according to standard microbiological protocol. All the specimens were processed for culture and identification of Methicillin resistance Staphylococcus aureus. All nasal carriage specimens were cultured on blood agar and Mannitol salt agar (Hi Media, New Delhi, India). S. aureus were identified and differentiated from related organisms as per conventional methods on the basis of colony morphology, Gram staining, catalase test, slide and tube coagulase, DNAse and mannitol fermentation following the standard procedures.[15] All the staphylococcus aureus isolates were tested for Methicillin resistance phenotypically by Oxacillin disc diffusion test, Oxacillin screen agar test, cefoxitin disc diffusion test [16] and further confirmed by mecA gene PCR. Staphylococcus aureus ATCC 25923 was used as MRSA Negative control where as Staphylococcus aureus ATCC 43300 was used as positive control strains.

Methods for detection of MRSA

1. Oxacillin disk diffusion test

Oxacillin disk diffusion test was performed on all isolates of S. aureus by using 1 ug of Oxacillin per disc on pre inoculated Mueller-Hinton agar plate added with 4% NaCl and incubated aerobically at 35ºC. The zone size was interpreted according to the CLSI guideline that is susceptible ≥13 mm and resistant ≤ 10 mm. [16]

2. Oxacillin screen agar

Mueller Hinton agar (MHA) plates containing 4% NaCl and 6 µg/ml of oxacillin were prepared. Plates were inoculated with 10 µL of 0.5 Mc Farland suspension of the isolate by streaking in one quadrant and incubated at 35^0C for 24 h. Plates were observed carefully in transmitted light for any growth. Any growth after 24 h was considered as Oxacillin resistant S. aureus. [16]

3. Cefoxitin disc diffusion test

Cefoxitin disc diffusion test was done by using 30 µg disc of cefoxitin on pre inoculated Muller Hinton agar plate for all isolates of S. aureus. The plates were incubated at 37°C for 18 to 24 hrs and zone diameters were measured. Zone diameters ≤19mm was reported as Methicillin resistant and zone diameters ≥22mm was considered as Methicillin sensitive. [16] Cefoxitin disc diffusion test by Kirby Bayer method is shown in figure- 1.

4. Detection of *Mec A* gene by PCR

Detection of *mecA* gene in S.aureus isolates were considered as the reference gold standard to compare sensitivity, specificity and rapidity of other phenotypic methods used in this study. PCR was performed according to the protocol used earlier by Tiwari et al, 2008. [17] The bacterial DNA was extracted by spin column method as per manufacturer instruction. 5 µl of the extracted DNA was transferred to 20 µl of PCR amplification mixture consisting of 2.5 µl of PCR buffer, 2.5 µl of MgCl2, 1.25U of *Taq* polymerase, 4 µl of dntps and 1 µl of each primer. The primer used in this study was reported earlier by Daniel J. Geha et al,[18] prior to blast on NCBI database. Forward primer was *mecA* 1 (5´ GTAGAAATGACTGAACGTCCGATAA 3´) and reverse primer was *mecA* 2- (5´ CCAATTCCACATTGTTTCGGTCTAA 3´). PCR was performed as per condition used earlier by Tiwari et al,[17] & Daniel J. Geha et al [18] with Initiation at 94°C for 4 min, followed by 30 cycles of denaturation at 45 sec at 94°C, annealing at 50°C for 45 s , and extension for 60 s at 72°C, with a final extension step at 72°C for 2 min. 10 µl of the PCR product of isolates was loaded in 2% agarose gel in TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) containing 0.5 µl /ml of ethidium bromide and visualized by using UV transilluminator at 300 nm. A product of 310 bp size was considered as positive for mec-A gene. (Figure 1)

STATISTICAL ANALYSIS

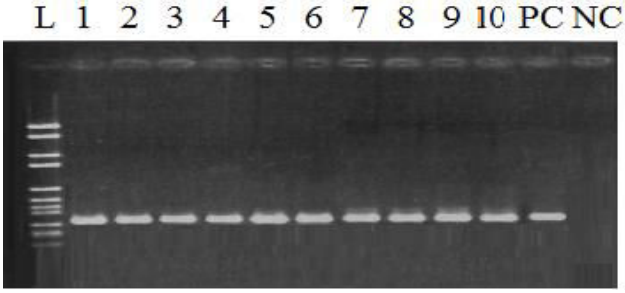
K2 test were used for evaluating association & kappa concordance measures was used levels of concordance of the data respectively.

RESULTS:

Nasal swab samples from 220 HIV seropositive patients (cases) and 220 healthy controls were collected with the help of sterile cotton swab. All the samples (cases & controls) were screened for *S. aureus* colonization. 43.64% isolates were confirmed to be S. aureus Out of 220 HIV seropositive patients among whom 18.75% were identified as MRSA and 81.25% as MSSA. Similarly among HIV negative patients; 21.82% isolates were

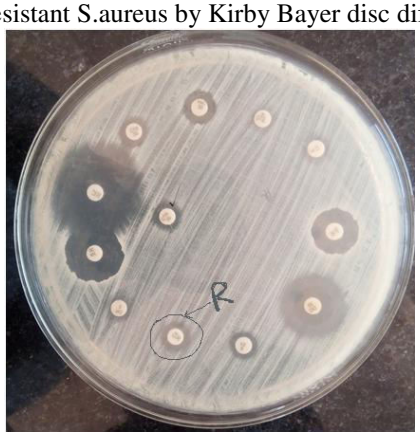
confirmed to be S. aureus among which 6.25% isolates were identified as MRSA while 93.75% were MSSA. In this way the *S.aureus* colonization was observed as 32.7% overall while, 14.58% MRSA was confirmed by PCR by amplification of 310 bp size mec-A gene product. **(Figure 2)** Detection of Methicillin Resistant S. aureus by different methods is summarized in **Table-1.** The sensitivity and specificity of the three phenotypic tests for MRSA detection was compared with genotypic test, taken mecA gene PCR as Gold standard; are given in **Table-2.** Cefoxitin disc diffusion showed 100% specificity (95% CI; 97.05% - 100.00%), 100% sensitivity (95% CI; 83.89% -100.00%) and 100% accuracy (95% CI; 97.47% to 100.00%) while comparing with gold standard mecA gene PCR.

Figure 2: Showing PCR product of 310 bp of mecA gene.



(L- Ladder, 1to10 - samples, PC- positive control, NC- Negative control)

Figure 1: Cefoxitin Resistant S.aureus by Kirby Bayer disc diffusion method



Zone of inhibition ≤ 21 mm by Cefoxitin denoted Methicillin Resistance.

Table- 1: Detection of Methicillin Resistant S. aureus by different methods as compared with mecA gene PCR.

mecA PCR		Oxacillin Disc Diffusion Method		Oxacillin Agar Screening Method		Cefoxitin Disc Diffusion Method	
n=144		Positive	Negative	Positive	Negative	Positive	Negative
Positive	21	16	5	20	1	21	0
Negative	123	2	121	0	123	0	123

Table- 2: Comparison of phenotypic methods for detection of MRSA (taking mecA gene PCR as Gold standard)

Statistical tool	Oxacillin Disc Diffusion Method		Oxacillin Agar Screening Method		Cefoxitin Disc Diffusion Method	
	Value	95% CI	Value	95% CI	Value	95% CI
Sensitivity	76.19 %	52.83% to 91.78%	95.24%	76.18% to 99.88%	100.00%	83.89% to 100.00%
Specificity	98.37 %	94.25% to 99.80%	100.00%	97.05% to 100.00%	100.00%	97.05% to 100.00%
Positive Likelihood Ratio	46.86	11.61 to 189.12	--	--	--	--
Negative Likelihood Ratio	0.24	0.11 to 0.52	0.05	0.01 to 0.32	0	--
Disease prevalence	14.58 %	9.26% to 21.42%	14.58%	9.26% to 21.42%	14.58%	9.26% to 21.42%
Positive Predictive Value	88.89 %	66.47% to 97.00%	100.00%	--	100.00%	--
Negative Predictive Value	96.03 %	91.84% to 98.11%	99.19 %	94.78% to 99.88%	100.00 %	--
Accuracy	95.14%	90.24% to 98.02%	99.31%	96.19% to 99.98%	100.00%	97.47% to 100.00%

DISCUSSION:

Staphylococcus aureus is one of the most important bacterial pathogen in the hospital as well as in community settings. Staphylococcus aureus infections account for significant morbidity in human immunodeficiency virus (HIV)-infected patients. Increase in Methicillin resistance among *Staphylococci may cause* great difficulty in managing such infections.[3] Hence, an accurate and rapid detection of Methicillin resistance is essential not only to choose appropriate antibiotic but also to control the spread of MRSA.

There are many phenotypic methods to detect MRSA but, they are time taken and vary in sensitivity and specificity. Currently, detection of *mecA* gene is considered as gold standard for MRSA detection. [19-21] However, molecular methods in routine laboratory are not affordable. Therefore it is necessary to develop a rapid, affordable, accurate and sensitive phenotypic method for detection of MRSA. Recent studies indicate that disc diffusion testing using cefoxitin disc is far superior to most of the currently recommended phenotypic methods like Oxacillin disc diffusion method and Oxacillin screen agar method. It is now an accepted method for the detection of MRSA by CLSI guideline.[16] The accurate and early detection of Methicillin resistance is important in the prognosis of MRSA associated infections. In this study, we have done a comparative analysis of different methods for detection of MRSA.

The sensitivity and specificity of the three phenotypic tests for MRSA detection was compared with genotypic test, taken mecA gene PCR as Gold standard; are given in **Table-2.**

In this study, 32.7% S.aureus were observed over all among which 14.58% nasal isolates were MRSA by mecA gene PCR. Cefoxitin disc diffusion showed 100% specificity (95% CI; 97.05% - 100.00%), 100% sensitivity (95% CI; 83.89% -100.00%) and 100% accuracy (95% CI; 97.47% to 100.00%) while comparing with gold standard mecA gene PCR. Whereas, the sensitivity and specificity was 95.24% (95% CI- 76.18% to 99.88%) and 100.00% (95% CI- 97.05% to 100.00%) respectively for Oxacillin screen agar; was not so accurate than Cefoxitin disc diffusion method. Results of cefoxitin disc diffusion test was in concordance with *mecA* gene PCR as shown in Table 2 and thus we recommend the cefoxitin disk diffusion method as an alternative to PCR for detection of MRSA in resource limited laboratories. Cefoxitin disc diffusion method is rapid and cost effective too. The similar finding was also reported by various authors. [19-21]

However, Oxacillin screen agar method for Methicillin resistance was reported the best screening tool having sensitivity 92.15% & specificity 90.90% for by Kunsang O Bhutia et a.[23] Similarly Latex agglutination test **was reported** to be the best & reliable method for Methicillin detection (100% sensitivity & specificity) by K.M. Mohanasoundaram et al.[22] **While Panda et al** [19] **Proposed** another test of high sensitivity and specificity like E-test (98.3%, 100%) should combine cefoxitin disc diffusion to confirm MRSA.

LIMITATIONS:

The study could not found any correlation between HIV infection and MRSA screening methods or possible effect on MRSA detection because in control group only 3 MRSA isolates were obtained.

CONCLUSION:

Based upon the findings, we recommend that cefoxitin can be used as a surrogate marker to detect Methicillin resistance S. aureus in routine susceptibility testing at 37^0 C for 18-24 hours. It can be used as an alternative tool for the mecA gene PCR.

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Source of support: Nil **Conflict of interest:** None declared